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Trans-Isolate Medium: a New Medium for Primary Culturing and Transport of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*

GLORIA W. AJELLO,[1]* JAMES C. FEELEY,[1] PEGGY S. HAYES,[1] ARTHUR L. REINGOLD,[2] GAIL BOLAN,[2] CLAIRE V. BROOME,[2] AND CATHERINE J. PHILLIPS[1]

Respiratory and Special Pathogens Laboratory Branch[1] and Respiratory and Special Pathogens Epidemiology Branch,[2] Division of Bacterial Diseases, Centers for Disease Control, Atlanta, Georgia 30333

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A diphasic medium, Trans-Isolate medium, was developed for the transport of primary cultures of cerebrospinal fluids from patients with bacterial meningitis. It consists of a charcoal-starch agar slant and soybean-casein digest-gelatin broth buffered at pH 7.2 with 0.1 M 3-(*N*-morpholino)propanesulfonic acid buffer. In the laboratory, this medium supported the growth and survival of stock cultures of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* for at least 3 months. Under field conditions in Africa, cerebrospinal fluids from patients suspected of having bacterial meningitis were inoculated directly onto plates of chocolate agar medium and into bottles of Trans-Isolate medium. An etiological agent was isolated from 52 spinal fluids by direct plating. After shipment to Atlanta, Ga., 2 to 4 weeks later, the same etiological agents were recovered from 38 bottles of Trans-Isolate medium.

Bacterial meningitis is an important cause of morbidity and mortality in developing countries. Cycles of epidemic meningitis have been well described in West Africa and may occur in other countries. Although it is frequently assumed that *Neisseria meningitidis* is responsible for most cases of epidemic meningitis, *Haemophilus influenzae* and *Streptococcus pneumoniae* are also common causes of endemic meningitis (1). Unfortunately, epidemiological and microbiological investigations of meningitis in these areas often are hampered by the lack of adequate laboratory facilities for primary isolation. Cultures from cerebrospinal fluids (CFSs) and other clinical materials often lose viability or become contaminated during shipment to a reference laboratory. For these reasons, the use of antigen detection methods has been suggested as an alternative. However, reagents are not commercially available for all serogroups of *N. meningitidis*; they are very expensive and may not be readily obtainable in developing countries. Culturing, on the other hand, is relatively inexpensive and provides isolates to be tested for antimicrobial susceptibilities as well as for markers for epidemiological characterization.

To conduct epidemiological studies in West Africa, a method of culturing CSF samples was needed which would permit growth of the etiological agents of bacterial meningitis (particularly *N. meningitidis*) and allow the cultures to survive adverse conditions in the field and during shipment. For these purposes, we developed and tested the Trans-Isolate (T-I) medium.

MATERIALS AND METHODS

Preparation of T-I medium. (i) Solid phase. Activated charcoal no. C5510 (Sigma Chemical Co., St. Louis, Mo.), 4.0 g; soluble starch (BBL Microbiology Systems, Cockeysville, Md.), 5.0 g; and Bacto-Agar (Difco Laboratories, Detroit, Mich.), 20.0 g were suspended in 1,000 ml of 0.1 M MOPS buffer (3-[*N*-morpholino]propanesulfonic acid) (Sigma) which had been adjusted to pH 7.2. These ingredients were heated to dissolve the starch and agar. With mechanical stirring to keep the charcoal in suspension, 15-ml quantities were dispensed into 60-ml Wheaton serum bottles. The bottles were covered with aluminum foil and autoclaved in metal baskets at 121°C for 20 min. The baskets were removed from the autoclave as soon as the sterilization cycle was completed, and the bottles, still in their baskets, were allowed to cool in a slanted position.

(ii) Liquid phase. Tryptic soy broth (TSB) (Difco), 60.0 g, and gelatin (BBL), 20.0 g were added to 1 liter of 0.1 M MOPS buffer adjusted to pH 7.2. The medium was heated to dissolve the gelatin and autoclaved at 121°C for 15 min. When cool, 20 ml of sterile supplement B (Difco) was added to permit the isolation of *H. influenzae*. A 15-ml portion of the broth was dispensed aseptically into each of the bottles containing the solid phase. The bottles were sealed with sterile rubber stoppers and aluminum caps (Fig. 1A).

For the isolation of *N. meningitidis* from potentially contaminated specimens, 20 ml of sterile, reconstituted VCN (vancomycin-colistin-nystatin) inhibitor (BBL) may be added per liter of liquid phase, but this combination of antimicrobial agents precludes the recovery of *S. pneumoniae* or *H. influenzae*.

Modification for transport. Screw-cap bottles (0.5 oz [ca. 15 ml]) were substituted for the 60-ml serum bottles; the solid and liquid phases of the medium were dispensed in 5.0-ml amounts, and autoclavable permeable membrane (PM) screw caps (size 18-451; Biochemical Polymers, Leominster, Mass.), which had been shortened by grinding on a lathe to a length of ca. 11 mm, were used to close the bottles (Fig. 1C).

Laboratory experiments. A series of laboratory experiments was performed. In experiment 1, we compared the growth and survival of *N. meningitidis* organisms in sealed and vented bottles (Fig. 1A and B) at 25 and 35°C. A stock strain of *N. meningitidis*, group A, isolated in 1981 from a case of epidemic meningitis in Mali, Africa, was used to test the new medium which was prepared in 60-ml serum bottles without VCN or supplement B. Organisms grown on a

* Corresponding author.

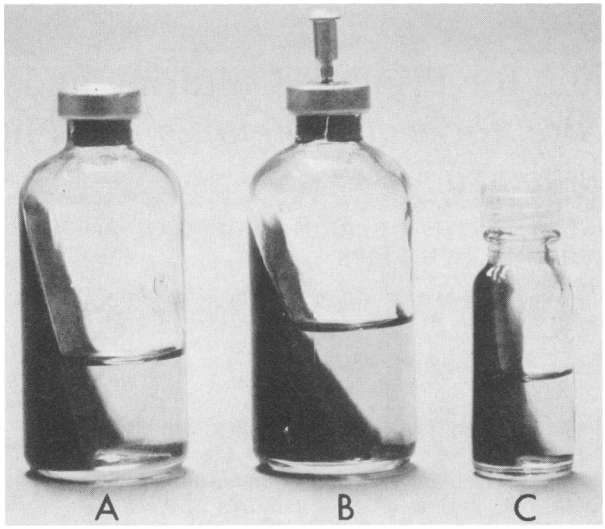

FIG. 1. T-I medium in serum bottles that were sealed (A) or vented with a sterile cotton-plugged needle (B). Screw-cap bottle with shortened PM cap (C).

chocolate agar plate for 24 h in a 35°C incubator with an atmosphere of 2.5% CO_2 in air were suspended in phosphate-buffered saline (pH 7.2), at a density corresponding to a 0.5 McFarland standard. Dilutions containing ca. 10^1 to 10^3 CFU per ml were made, and 1-ml samples were injected into each bottle. The bottles were incubated sealed or vented with cotton-plugged hypodermic needles at 25 ± 2°C and at 35°C. In experiment 2, we evaluated growth and survival in vented and sealed bottles incubated at 42°C. Two hundred CFU were inoculated into each of 10 bottles (5 vented and 5 sealed) incubated at 42°C. In experiment 3, the effect of holding mature, healthy cultures in bottles (5 vented and 5 sealed) at 4°C was examined. The serum bottles with visible turbidity were sampled at weekly intervals by removing 0.05 ml of the broth with a syringe and needle for spreading onto chocolate agar plates. Unless otherwise noted, all plates were incubated at 35°C in air supplemented with 2.5% CO_2. In experiment 4, we compared the growth and survival of *N. meningitidis* in T-I medium with and without VCN and supplement B. In experiment 5, we compared the survival of recently isolated *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* strains in 15-ml bottles closed with solid screw caps versus PM caps. The T-I medium was prepared with supplement B. Three different inoculum sizes were used. The bottles were sampled at weekly intervals by using a pipette.

Field testing of the medium. The T-I medium in 15-ml bottles with supplement B was field tested in Upper Volta with both CSF and nasopharyngeal (N-P) specimens. The CSF specimens were collected in a hospital in Ouagadougou, Upper Volta, from patients suspected of having bacterial meningitis, and were usually delivered to our field laboratory within 3 h; however, delivery was occasionally delayed for 16 h. Approximately 0.05 ml of each CSF sample was inoculated onto a chocolate agar plate which was incubated at 37°C in a candle extinction jar humidified with a moistened paper towel. Bottles of T-I medium were inoculated with 0.25 to 0.5 ml of CSF and held at room temperature (15 to 35°C) for 1 to 3 weeks. Bottles were subcultured before and after shipment to Atlanta, Ga.

The N-P specimens used to test the T-I medium were collected from 88 students living at a technical college in Ouagadougou where an outbreak of meningococcal meningitis was occurring. A calcium alginate swab with a flexible aluminum shaft was used to obtain an N-P specimen from each student. Each swab was dipped into a bottle of T-I medium containing 1.0% VCN and was then discarded. The bottles were incubated at 37°C until turbidity developed, usually in 1 to 3 days, whereupon a loopful of the broth was streaked onto a chocolate agar plate. After 24 and 48 h of incubation in humidified candle jars, the plates were examined for the presence of *N. meningitidis* colonies. Pure isolates were then inoculated into new bottles of T-I medium with VCN and shipped to Atlanta.

Colonies on the chocolate agar plates were presumptively identified as *N. meningitidis* on the basis of their morphological appearance, oxidase and *o*-nitrophenyl-β-D-galactopyranoside reactions, and agglutinability in specific group antisera. *H. influenzae* isolates were selected also by colony appearance and were tested for X- and V-factor requirements and for agglutination in specific antisera. Colonies having the typical appearance of *S. pneumoniae* were tested for optochin susceptibility. Definitive biochemical testing, serological typing, and antimicrobial susceptibility tests were performed in Atlanta.

RESULTS

Laboratory experiments. In experiment 1, all of the 60-ml bottles of T-I medium (without supplements) inoculated with the stock strain of *N. meningitidis* and incubated at 35°C showed turbidity in the broth phase within 24 h and growth on the slants within 3 to 5 days; the bottles held at ca. 25°C showed visible growth 1 or 2 days later. After incubation for 5 days at either temperature, samples from all of the T-I bottles yielded heavy, confluent growth on chocolate agar plates within 18 h. Venting the bottles enhanced the survival of *N. meningitidis* at all temperatures except possibly at 4°C (Table 1). At 25°C, *N. meningitidis* in vented bottles survived for a mean of >24 weeks versus a mean of 1.4 weeks in sealed bottles. At 35°C, *N. meningitidis* survived for a mean of 15.9 weeks in vented bottles and only for a mean of 1.6 weeks in sealed bottles. Removal of the vents from the bottles resulted in negative subcultures within 1 week.

In experiment 2, heavy growth occurred in two of five vented bottles incubated at 42°C, whereas no growth appeared in the sealed bottles held at this temperature. One of the vented bottles was positive on subculture for 5 weeks, whereas the other contained viable organisms when subcultured after 17 weeks (Table 1). Subcultures on chocolate agar plates grew luxuriantly at 35°C but did not grow at 42°C.

Experiment 3 showed that venting the bottles did not enhance the survival of *N. meningitidis* when held at 4°C. Organisms in 10 vented bottles held at 35°C for 28 days and subsequently refrigerated, 5 vented and 5 with the vents removed, remained viable for 3 to 7 weeks at 4°C (Table 1).

In experiment 4, we found that the addition of supplement B or VCN (or both) to the T-I medium appeared to have no effect on the growth and length of survival of *N. meningitidis*.

N. meningitidis grew well at 35°C in T-I medium prepared in 15-ml screw-cap bottles (Table 2). Cultures in bottles with PM caps remained viable throughout the duration of the experiment (5 weeks), but cultures in bottles with solid screw caps died in 1 to 2 weeks. *S. pneumoniae* and *H. influenzae* cultures survived for at least 5 weeks in bottles with either type of cap. Bottles inoculated with <10 CFU of

TABLE 1. Summary of laboratory experiments 1–3 on the growth and survival of *N. meningitidis* in 60-ml bottles of T-I medium at various temperatures

Expt no.	Temp (°C)	Vented bottles[a]			Sealed bottles		
		No. of bottles	Wks of survival		No. of bottles	Wks of survival	
			Range	Mean		Range	Mean
1	25	3	>24	>24	12	1–2	1.4
	35	13	6–25	15.9	4	1–2	1.6
2	42	5	<1[b]–17	4.4	5	<1	<1
3	4[c]	5	3–4	3.2	5	4–7	5.4

[a] Vented with a sterile cotton-plugged needle.
[b] No turbidity was noted in bottles surviving <1 week.
[c] Incubated vented at 35°C for 4 weeks before transfer to refrigerator.

S. pneumoniae or *H. influenzae* did not always survive as long as bottles inoculated with >10 CFU.

T-I medium with supplement B also supported growth, in the liquid phase only, of isolates of *S. pneumoniae* and *H. influenzae* b at 35°C and at room temperature. *H. influenzae* grew at 42°C, but *S. pneumoniae* did not. Cultures of both organisms survived for at least 3 months in vented and sealed bottles.

Field trials. Each of 89 CSF specimens was simultaneously inoculated onto a chocolate agar plate and into a 15-ml T-I bottle with a PM cap. The total of 52 etiological agents recovered by direct plating included 26 strains of *N. meningitidis* serogroup A, 13 of *H. influenzae* b, and 13 of various serotypes of *S. pneumoniae* (Table 3). Shortly before shipment to Atlanta, the T-I bottles showing growth were sampled, and etiological agents were isolated from 46 (88%) of them. The same etiological agents that were found on plates grew in the bottles except three that did not grow (two *H. influenzae* and one *S. pneumoniae*) and three (two *N. meningitidis* and one *H. influenzae*) that were overgrown by contaminants. No etiological agents from CSF were cultured from bottles that were not cultured also on plates. During shipment, eight more etiological agents were overgrown by contaminants. Consequently, 38 (73%) of the 52 etiological agents were recovered in the reference laboratory from the primary cultures in T-I bottles.

Of 88 T-I bottles containing VCN that were inoculated with N-P swabs from the students, 29 (33%) yielded cultures of meningococci with few contaminants when subcultured to chocolate agar. After inoculation into fresh T-I bottles and shipment to Atlanta, 28 cultures were recovered.

TABLE 2. Survival of laboratory strains of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* in T-I bottles

Organism	Inoculum[a]	Survival (wks) in bottles with:	
		PM caps	Solid caps
N. meningitidis	470	≥5	1
	36	≥5	2
	5	≥5	2
S. pneumoniae	57	≥5	≥5
	5	≥5	3
	<1	≥5	0
H. influenzae	1,150	≥5	≥5
	155	2	≥5
	9	1	3

[a] Number of CFU inoculated into bottles which were incubated at 35°C.

DISCUSSION

No single medium, to our knowledge, has ever been reported to function adequately as a growth, holding, and transport medium for the etiological agents of bacterial meningitis. Primary isolates, cultured on plates, are generally subcultured on a medium known to support growth in tubes or vials suitable for shipping. Media, such as cystine-tryptic agar (CTA), Transgrow medium, and chocolate agar have been used for transporting cultures intercontinentally with varying degrees of success. For example, two of nine (22%) isolates of *N. meningitidis* from Africa were recovered from CTA cultures after 14 days in transit (P. Hayes, unpublished data), and 43% of fresh *N. meningitidis* subcultures shipped from Brazil in Transgrow medium were viable after 7 days (R. Munford, unpublished data). From chocolate agar slants, 10 of 14 (71%) *N. meningitidis* isolates, freshly subcultured 24 h before shipment from Africa, were recovered after 9 days. Two cultures of *H. influenzae* that were shipped at the same time also were recovered, but all of six cultures of *S. pneumoniae* were nonviable (C. Phillips, unpublished data). A blood-free, diphasic medium developed by Sanborn was used in Egypt for culturing the etiological agents of bacterial meningitis from CSFs (4). Although this medium was found superior to agar plates or broths for the primary culture of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* organisms in a hospital laboratory, its utility as a transport medium had not been determined. We doubted that the small amount of starch contained in Sanborn medium would be sufficient to protect the cultures of CSF pathogens for long periods under adverse conditions. Therefore, we elected to evaluate TSB with the addition of

TABLE 3. Recovery of etiological agents of bacterial meningitis from 52 CSF specimens

Etiological agent	Culture method		
	Direct plate[a]	T-I Bottles[b]	
		In Africa	In Atlanta
N. meningitidis	26	24 (92)	21 (81)
S. pneumoniae	13	12 (92)	12 (92)
H. influenzae	13	10 (77)	5 (38)

[a] Done only in Africa.
[b] T-I medium prepared with supplement B and without VCN. Number recovered from bottles expressed as percentage of number recovered by direct plating is given in parentheses. Difference in totals between those done in Africa and those done in Atlanta represents the loss of eight etiological agents due to contamination of the bottles during shipment.

detoxifying and protective substances as a more suitable medium for our purposes.

Because activated charcoal and starch appear to neutralize different inhibitory substances (3), we selected both as detoxifying agents. They were incorporated into the agar slant to avoid clouding the broth, which would otherwise prevent visualization of growth. Gelatin was added to the broth because it has been shown to preserve many species of bacteria, possibly by forming a protective coat on the cell surface (5). The liquid phase was prepared in double strength so that nutrients would diffuse through the interstices of the slant and support growth on the agar as well. A 0.1 M solution of MOPS, an organic buffer, was chosen as the solvent because of its nontoxicity and high buffering capacity at the optimal pH for the meningococcus. With this combination of a wholly autoclavable solid medium and a transparent broth, preparation of the medium is not too cumbersome and growth can be detected easily.

The new medium has been shown to be capable of allowing heavy growth from inocula containing small numbers of meningococci. Since little change in the pH of the medium occurred in a week or in the months after inoculation, the buffered medium appeared to protect the cultures from destruction by highly acid or highly alkaline metabolic by-products. Our results also indicated that the medium protected *N. meningitidis* at temperatures as high as 42°C and as low as 4°C and that the presence of phosphate in the TSB did no appreciable harm to the organisms.

The fact that *N. meningitidis* is obligately aerobic only partially explains why cultures remained viable in vented bottles, whereas cultures in sealed bottles died within a few days. The rapid die-off of sealed cultures may be due, in part, to exhaustion of the air supply or to an accumulation of volatile toxic products which are unable to escape. The autoclavable PM screw caps, which permit the interchange of respiratory and probably other gases, also served as a means of shipping the bottles without leakage. The use of small containers to accomodate the caps was also advantageous in saving space and in cutting the costs of materials, reagents, and transportation. Although the bottles contained one-third the amount of medium originally tested, this did not affect the length of survival of the etiological agents.

Contamination was the chief cause of failure to recover an etiological agent from the T-I bottles inoculated with positive cerebrospinal fluids. In many developing areas, CSFs are not collected under aseptic conditions. They can easily become contaminated with environmental organisms unless they are drawn with great care. The cause of the contamination that developed in eight apparently pure cultures during shipment could not be traced to a particular source.

Antimicrobial inhibitors added to the liquid phase of the bottles may be useful to decrease the occurrence of contamination. There is little doubt that VCN inhibitor aided in the recovery of *N. meningitidis* within 1 to 3 days after bottles were inoculated with mixed N-P flora. However, after prolonged holding periods, VCN has not always prevented overgrowth by some environmental bacteria. The formulation of different combinations of antimicrobial agents to better protect *N. meningitidis* or to suppress contamination in bottles used for culturing *H. influenzae* or *S. pneumoniae* will be investigated.

Despite the problem of sporadic contamination, 73% of the T-I bottles inoculated with CSFs 2 to 4 weeks previously yielded a primary culture of *N. meningitidis*, *H. influenzae*, or *S. pneumoniae* at their final destination. This method of culturing CSFs can provide opportunities for the study of outbreaks of bacterial meningitis in remote areas. Using precautions to avoid contamination, local health care personnel can inoculate the bottles and hold them for several days, or longer, at ambient temperatures for transport to a microbiological laboratory. The ability to recover, identify, and characterize the etiological agents of bacterial meningitis in these areas can furnish physicians and public health officials with valuable information on which to base control measures.

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